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Decreased Cerebrovascular Brain-Derived Neurotrophic Factor–Mediated Neuroprotection in the Diabetic Brain

Deepti Navaratna,¹ Shu-zhen Guo,¹ Kazuhide Hayakawa,¹ Xiaoying Wang,¹ Chiara Gerhardinger,² and Eng H. Lo¹

OBJECTIVE—Diabetes is an independent risk factor for stroke. However, the underlying mechanism of how diabetes confers that risk is not fully understood. We hypothesize that secretion of neurotrophic factors by the cerebral endothelium, such as brain-derived neurotrophic factor (BDNF), is suppressed in diabetes. Consequently, such accrued neuroprotective deficits make neurons more vulnerable to injury.

RESEARCH DESIGN AND METHODS—We examined BDNF protein levels in a streptozotocin-induced rat model of diabetes by Western blotting and immunohistochemistry. Levels of total and secreted BDNF protein were quantified in human brain microvascular endothelial cells after exposure to advanced glycation end product (AGE)-BSA by enzyme-linked immunosorbent assay and immunocytochemistry. In media transfer experiments, the neuroprotective efficacy of conditioned media from normal healthy endothelial cells was compared with AGE-treated endothelial cells in an in vitro hypoxic injury model.

RESULTS—Cerebrovascular BDNF protein was reduced in the cortical endothelium in 6-month diabetic rats. Immunohistochemical analysis of 6-week diabetic brain sections showed that the reduction of BDNF occurs early after induction of diabetes. Treatment of brain microvascular endothelial cells with AGE caused a similar reduction in BDNF protein and secretion in an extracellular signal–related kinase-dependent manner. In media transfer experiments, conditioned media from AGE-treated endothelial cells were less neuroprotective against hypoxic injury because of a decrease in secreted BDNF.

CONCLUSIONS—Taken together, our findings suggest that a progressive depletion of microvascular neuroprotection in diabetes elevates the risk of neuronal injury for a variety of central nervous system diseases, including stroke and neurodegeneration. *Diabetes* 60:1789–1796, 2011

Diabetes mellitus significantly elevates the risk for a variety of neurologic diseases, including stroke (1–3). Age-adjusted incidence rates suggest that diabetic patients are three times more likely to have a stroke compared with nondiabetic patients, a disparity that is seen across multiple racial/geographic groups (4–7). In addition, diabetes is associated with more severe strokes, in-hospital mortality, and slower recovery compared with nondiabetic individuals (8–11). Diminished cognitive abilities are found in patients with type 1 diabetes,

whereas type 2 diabetes is known to also affect learning and memory (12,13). Many population-based studies have found an association between diabetes and an increased risk of developing Alzheimer's disease and vascular dementia (14,15).

How does diabetes confer this elevated risk for progressive neuronal injury? In this study, we explored the hypothesis that cerebrovascular neurotrophic protection is reduced in diabetes, thereby causing progressive neuronal dysfunction. Neurons do not exist in isolation, and symbiotic trophic coupling mechanisms exist between cerebral microvasculature and neurons (16–21). Recent studies suggest that endothelial cells serve endocrine functions in the brain by secreting neuroprotective factors, such as brain-derived neurotrophic factor (BDNF) (22,23). In this study, we investigate the implications of decreased cerebrovascular BDNF–mediated neurotrophic function in diabetes in an effort to examine the role of vascular dysfunction in diabetes complications of the brain.

RESEARCH DESIGN AND METHODS

Diabetic rat model of diabetes. All experiments were performed following an institutionally approved protocol in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Diabetes was induced with streptozotocin (STZ) (57.5 mg/kg body wt) dissolved in citrate buffer, pH 4.5, through a tail-vein injection. Induction of diabetes was verified after 3 days, and rats with blood glucose concentrations >250 mg/dL were included in these studies. Body weight was measured three times per week, and 3–4 units of neutral protamine Hagedorn insulin (0.5 IU) was administered subcutaneously, as needed, to prevent weight loss and ketosis. The diabetic rats were killed, and brains were collected after perfusion along with age-matched controls. Cerebral cortex was dissected from whole brains for microvessel-enriched fractions and stored at –80°C before fraction preparation. At the time of death, blood was obtained by cardiac puncture for estimation of glycosylated hemoglobin (Glyco-Tek Affinity Kit, Helena Laboratories, Beaumont, TX).

Brain microvessel preparation by dextran gradient centrifugation. Isolation of microvessel-enriched fractions from rat tissue was performed as described by Wu et al. (24) and Galea and Estrada (25). Briefly, brain tissues were collected from rats after PBS reperfusion. The cortical gray matter was dissected and rolled on filter paper to remove the large blood vessels and then homogenized with PBS and centrifuged. After washing with PBS, the pellet was resuspended in four volumes of 18% dextran and centrifuged at 1,500g for 20 min. The new pellet was saved, and the remaining tissue was reprocessed twice similarly. All three pellets were pooled, washed again with PBS, and lysed in lysis buffer (Cell Signaling Technology, Danvers, MA) with proteinase inhibitors for immunoblotting. Microvessel-enriched fractions in all experiments were not pooled and were derived from a single animal cortex.

Immunohistochemical procedures. For immunohistochemistry, whole brain sections (2-mm thick) were prepared from frozen rat brains. Sections were kept frozen at –80°, after which they were air-dried, fixed in ice-cold acetone, blocked with 5% BSA, and probed with an anti-BDNF rabbit polyclonal antibody (Millipore, Billerica, MA). For immunocytochemistry, cells were seeded onto gelatin-coated glass coverslips and maintained in complete media. Cells were fixed in ice-cold ethanol, rinsed with 1× PBS, blocked with 5% BSA, and probed with an anti-BDNF antibody (Millipore) or anti-CD31 antibody (BD Biosciences, San Diego, CA).

Cell culture and treatments. Primary human brain microvessel endothelial cells were purchased from Cell Systems Corporation (Kirkland, WA), mostly

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TABLE 1

Body weight and glycated hemoglobin of study rats (6 months' diabetes duration)

| | N | Body weight at start (g) | Body weight at end (g) | Glycated hemoglobin (%) |
|-------------|---|-----------------------------|---------------------------|----------------------------|
| Nondiabetic | 4 | 260.5 ± 48.5 | 606.7 ± 58.1 | 4.2 ± 0.22 |
| Diabetic | 4 | 253.2 ± 50.9 | 385.7 ± 30.9 | 14.2 ± 1.6* |

Percentage of glycated hemoglobin in blood samples of 6-month diabetic rats was significantly higher than in age-matched nondiabetic rats. Values are mean ± SD. Statistical analysis was performed with unpaired *t* test. **P* < 0.05 compared with nondiabetic rats.

derived from a heterogeneous mix of rapidly autopsied human brains obtained within a few hours after death. We used cells between passages 5 and 10 at ~80% confluence for all studies. The endothelial cells were maintained in complete MCDB-131 medium with L-glutamine, 1 g/L D-glucose, and 10% FBS (Vec Technologies, New York, NY), and seeded onto human gelatin-coated plates for all experiments. Primary mouse embryo cortex neurons were seeded on poly-D-lysine-coated plates and maintained with Neurobasal media plus 2% B27, 0.5 μmol/L glutamine, and penicillin/streptomycin (Invitrogen, Carlsbad, CA). Neurons were used for conditioned media-transfer experiments between days 8 and 10. All treatments with BSA, advanced glycation end product (AGE)-BSA, and the MEK/extracellular signal-related kinase (ERK) inhibitor U0126 were performed in 1% serum MCDB media supplemented with L-glutamine and 4.5 g/L D-glucose. AGE-BSA and U0126 were procured from Calbiochem (San Diego, CA).

ELISAs, cytotoxicity assay, immunoblotting. The ELISA kit for BDNF was obtained from Promega (Madison, WI). MTT assay and LIVE/DEAD viability/cytotoxicity assay were used to assess cell death. For immunoblotting, the primary antibodies were obtained from Cell Signaling Technology (BDNF

antibody), Biomol/Enzo Life Sciences (neuron-specific enolase; Plymouth Meeting, PA), and Promega (total and pERK antibodies). All secondary antibodies were obtained from Pharmacia (New York, NY).

Hypoxia and media transfer experiments. Primary endothelial cells were treated with or without AGE-BSA for 48 h, before the conditioned media were transferred to neurons. Conditioned media collected from endothelial cells were transferred to primary mouse cortical neurons cultured overnight in serum-free Neurobasal media minus B27 and antibiotics. Neurons were then placed in a hypoxic chamber (Billups-Rothenberg, Inc., Del Mar, CA). The chamber with cells was perfused with mixed atmosphere (90% nitrogen, 5% carbon dioxide, and 5% hydrogen) for 30 min and then sealed and placed at 37°C for indicated time periods of hypoxia. After 48 h, cells were removed from the chamber and changed to fresh Neurobasal serum-free media, maintained in a regular CO₂ incubator for reoxygenation. Control cultures were incubated under normoxic conditions for equivalent durations.

Statistical methods. Quantitative data from all experiments were analyzed using the JMP-IN statistical software 7.0 (SAS Institute Inc., Cary, NC). Student *t* test was performed for two-group comparisons. One-way ANOVA was performed for multiple group comparisons followed by Tukey-Kramer tests for pairwise comparisons. Differences reaching *P* < 0.05 were considered significant.

RESULTS

BDNF expression is reduced in diabetic rat brain endothelium in vivo. Diabetes was induced in adult male SD rats by STZ injection. After 6 months of diabetes, the diabetic rats showed lower body weight and higher glycohemoglobin levels than the age-matched nondiabetic control rats (Table 1).

Diabetic and nondiabetic rat cerebral cortices were extracted, and microvessel-enriched fractions were prepared for Western blotting. These fractions were positive

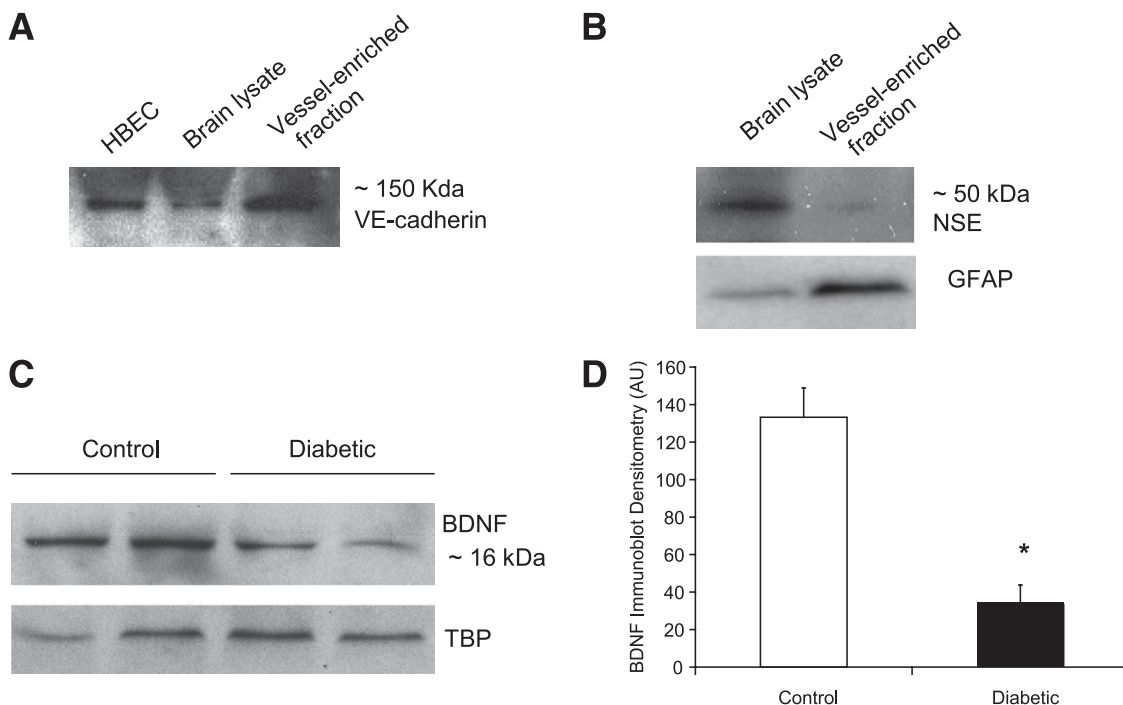


FIG. 1. BDNF protein is reduced in microvessel-enriched fractions of the diabetic rat cortex. Representative Western blots showing significant enrichment of endothelial components in microvessel-enriched fractions, as demonstrated by high levels of vascular endothelial-cadherin protein (A) and low levels of neuron-specific enolase and glial fibrillary acidic protein (B). BDNF levels are reduced in the microvessel-enriched fractions of 6-month diabetic rat cortex compared with age-matched nondiabetic rat cortex. TATA-binding protein is used as loading control (C). Densitometric quantitation of Western blots show that BDNF levels in microvessel-enriched brain fractions are significantly higher in age-matched nondiabetic rat cortex compared with 6-month diabetic rat cortex (D). **P* < 0.05 between control and diabetic rat cortices, *N* = 4 per group. GFAP, glial fibrillary acidic protein; HBEC, human brain endothelial cell; NSE, neuron-specific enolase; TBP, TATA-binding protein; VE, vascular endothelial.

for vascular endothelial cadherin, confirming their endothelial identity (Fig. 1A), whereas staining for neuron-specific enolase and glial fibrillary acidic protein was weak, suggesting that they were not overly contaminated with neurons or astrocytic components (Fig. 1B). A 16-kDa band corresponding to bioactive BDNF protein was detected in all fractions. BDNF levels were significantly reduced in microvessel-enriched fractions from diabetic rats compared with nondiabetic rats (Fig. 1C and D). To confirm our findings, 6-week normal and diabetic rat brains were sectioned and prepared for immunohistochemistry. BDNF staining was observed in the blood vessels of the cortex. BDNF levels appeared to be lower in diabetic endothelium compared with normal vessels (Fig. 2).

AGE-BSA treatment decreases BDNF levels in brain endothelial cells. Our *in vivo* data suggested that endothelial BDNF was suppressed in diabetic endothelium. To assess the mechanisms involved, we switched to an *in vitro* experimental model using AGE-BSA to mimic endothelial stress triggered during diabetes. Human microvascular brain endothelial cells were incubated with AGE-BSA for 48 h in serum-free media. This treatment caused a change in cell morphology, with changes in cell shape and loss of cell-cell contact (Fig. 3A).

After 48 h, BDNF protein levels in the cell lysates were assessed by Western blotting. AGE-BSA clearly induced a dose-dependent reduction in BDNF levels (Fig. 3B). Compared with normal BSA controls, BDNF levels were significantly reduced in the AGE-BSA-treated endothelial cells (Fig. 3C and D). No changes in cell viability were

noted, suggesting that these reductions in BDNF were not indirectly caused by nonspecific cytotoxicity (Fig. 3E).

Immunostaining confirmed our Western blot findings. In normal human brain endothelial cells, cytoplasmic BDNF staining was observable in the perinuclear zone and in vesicles being trafficked to the plasma membrane (Fig. 4A). In AGE-BSA-treated endothelial cells, there was a clear reduction in BDNF staining compared with untreated controls (Fig. 4A). Consistent with these immunostaining observations, AGE-BSA affected the secretion of BDNF into extracellular space. Standard enzyme-linked immunosorbent assays confirmed that conditioned media from brain endothelial cells contained BDNF. AGE-BSA-treated endothelial cells showed a significant decrease in secreted BDNF compared with untreated cells or controls treated with normal BSA (Fig. 4B).

AGE-BSA-induced reduction of BDNF is dependent on ERK/MAP kinase signaling. AGEs can trigger many intracellular signaling pathways. Because ERK/mitogen-activated protein (MAP) kinase is one of the major mechanisms that allow endothelial cells to respond to extracellular stimuli, we asked whether this signaling pathway might be involved in the phenomenon of AGE-BSA-induced downregulation of BDNF. Human brain endothelial cells were exposed to AGE-BSA for 48 h, and then cell lysates were probed for changes in ERK and phospho-ERK. Levels of phospho-ERK were significantly elevated in AGE-BSA-treated cells with no change in total ERK (Fig. 5A and B). Next, we tested the effects of the potent MEK/ERK inhibitor U0126 to provide pharmacologic evidence of causality. As expected, AGE-BSA decreased BDNF expression in brain endothelial cells. Blockade of ERK signaling with U0126 decreased phospho-ERK levels and significantly prevented this AGE-BSA-induced suppression of BDNF (Fig. 5C and D). Concomitantly, blockade of ERK signaling also interfered with the ability of AGE-BSA to decrease the secretion of BDNF into conditioned media (Fig. 5E). Taken together, these data suggest that the ability of AGE-BSA to suppress endothelial BDNF requires ERK/MAP kinase signaling.

BDNF-mediated neuroprotection is lost after AGE-BSA treatment in brain endothelial cells. Because AGE-BSA decreased the production of BDNF, we hypothesized that this would mean that “diabetic” endothelium would lose the ability to trophically protect neurons. First, we confirmed that AGE-BSA does not directly kill neurons (Fig. 6A). Next, media transfer experiments were performed whereby brain endothelial-conditioned media were added to primary mouse neurons subjected to hypoxia for 24 h. As expected, hypoxia killed neurons (Fig. 6B and C), and, as previously reported, conditioned media from normal healthy brain endothelial cells were significantly neuroprotective (Fig. 6B and C). In contrast, conditioned media from AGE-BSA-treated endothelial cells were no longer neuroprotective (Fig. 6A and B), consistent with their reduction in BDNF levels. Addition of exogenous BDNF back into the culture media restored protection and decreased neuronal death (Fig. 6B and C). Finally, the role of ERK signaling in the pathway was confirmed by U0126 experiments. Blockade of ERK with U0126 not only prevented endothelial BDNF suppression (Fig. 5D) but also restored neuroprotection (Fig. 6C).

DISCUSSION

Symptomatic strokes caused by large artery disease have garnered much attention in cerebrovascular investigations.

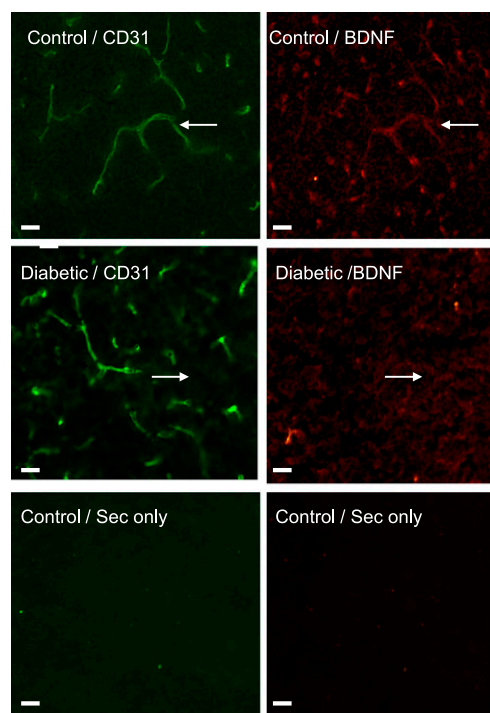


FIG. 2. BDNF expression is reduced in the diabetic brain endothelium. Representative images of cortical sections (2-mm thick) from 6-week diabetic and age-matched nondiabetic rats immunostained for BDNF. Blood vessels (arrows) are visualized by CD31 staining (left). Cortex microvessels from a diabetic rat show decreased BDNF immunofluorescence when compared with those of a nondiabetic rat (right). Magnification 40 \times . (A high-quality digital representation of this figure is available in the online issue.)

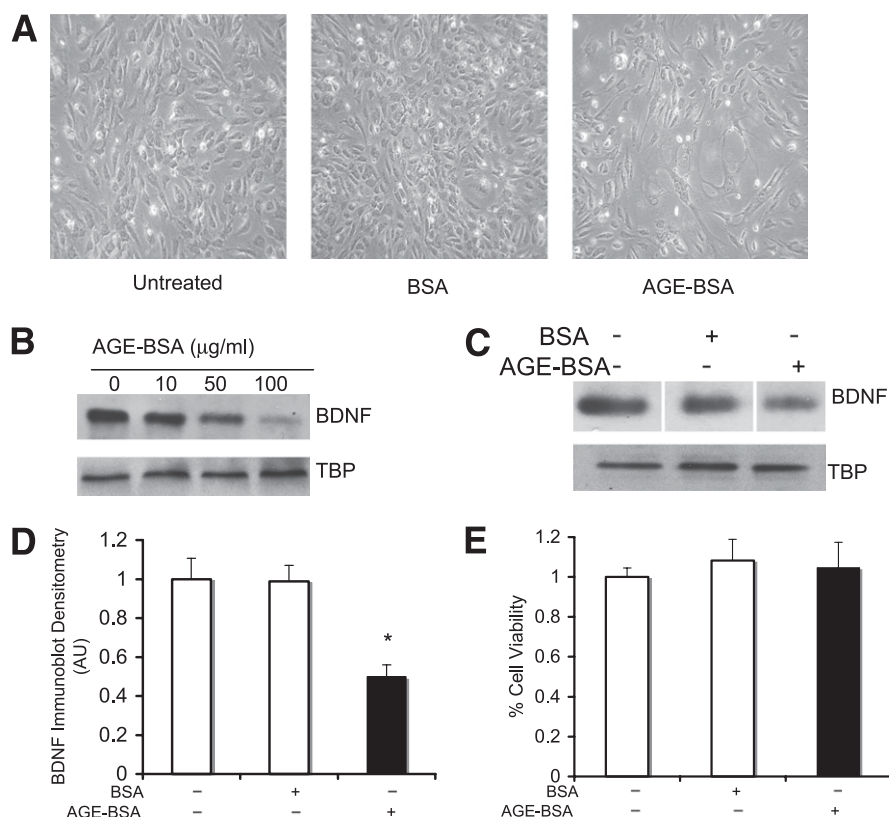


FIG. 3. AGE-BSA treatment decreases BDNF levels in endothelial cells. **A:** Cell morphology is altered by 48-h AGE-BSA treatment. Cells show decreased cell-cell contact and changes in cell shape. **B:** Representative Western blot shows that BDNF protein is reduced in cell lysates in a dose-dependent manner after AGE treatment (10–100 $\mu\text{g/ml}$). TATA-binding protein is used as loading control. **C:** Representative Western blot shows that exposure to 50 $\mu\text{g/ml}$ of AGE-BSA reduced BDNF protein. Treatment with 50 $\mu\text{g/ml}$ of unglycated BSA did not alter BDNF levels. Gel sections were derived from a single representative Western blot. **D:** Quantitative densitometry showing that AGE-BSA-treated endothelial cells expressed significantly lower BDNF compared with untreated cells or cells treated with normal BSA. * $P < 0.05$ between untreated and AGE-BSA treatment, $N = 3$ per group. **E:** AGE-BSA treatment (50 $\mu\text{g/ml}$) did not cause overt cytotoxicity as measured by the MTT assay, $N = 3$ per group. TBP, TATA-binding protein.

In contrast, small vessel dysfunction in the deeper layers of the brain cortex has received less emphasis, even though this comprises approximately one-third of all symptomatic strokes (26). This problem may be especially important for diabetic patients who present a higher incidence of lacunar stroke with recursive hemorrhagic episodes (6,27). Although the effects of acute hyperglycemia on brain injury are well documented in global and focal ischemia models (28), there is a bigger need to first study the effects of long-term diabetes on the brain. Emerging studies support the notion that preemptive vascular disease in diabetes affects ischemic brain injury before, during, and after stroke by modifying adaptive neovascularization responses and post-ischemic tissue remodeling (29,30). However, there is little clarity on baseline changes in cerebrovascular function in diabetes and the relative contribution of major diabetic stressors to cerebrovascular disease. Our findings suggest that cellular stress from AGEs leads to reduced cerebrovascular secretion of BDNF, a major mediator of neuroprotection.

A large body of evidence suggests that AGEs are central mediators of almost all diabetic vascular complications (31–34), beyond effects of prolonged hyperglycemia. A marked increase in deposition and accumulation of protein glycation end products in blood vessels has been linked to corresponding neuronal impairment, cognitive dysfunction, and dementia in several streams of investigation. In human

brain tissue, increased cerebrovascular carboxymethyl lysine-AGE staining was observed in people with diabetes and clinical dementia, strengthening the argument that vascular accumulation of AGEs correspond with clinical presentations of neuronal injury (35). Notably, an accumulation of AGEs in microvessels along with its receptor, RAGE, is reported to drive microvascular AGE-RAGE-mediated chronic inflammation in Alzheimer pathologies (36). In addition, administration of AGE-BSA significantly increased cerebral infarct size in a rat model of focal cerebral ischemia, suggesting that increased severity of stroke associated with diabetes might be characterized by AGE accumulation (37). In our study, treatment of primary brain microvascular cells with AGE-BSA significantly suppressed the production of BDNF via ERK/MAP kinase signaling pathways, whereas treatment with high glucose alone did not (data not shown). Subsequently, because of the loss of BDNF, endothelial cells exposed to AGE-BSA were no longer able to protect neuronal cultures against hypoxic injury. Although our *in vitro* model of AGE-BSA treatment cannot be equated to *in vivo* diabetic conditions, it is useful in assessing the direct contribution of AGEs to neurotrophic reduction observed *in vivo*.

We chose to study BDNF levels at 6 weeks and 6 months of diabetes as a suitable time window to characterize early chronic injury in diabetes. Several studies have focused on this early duration of diabetes to capture adaptive

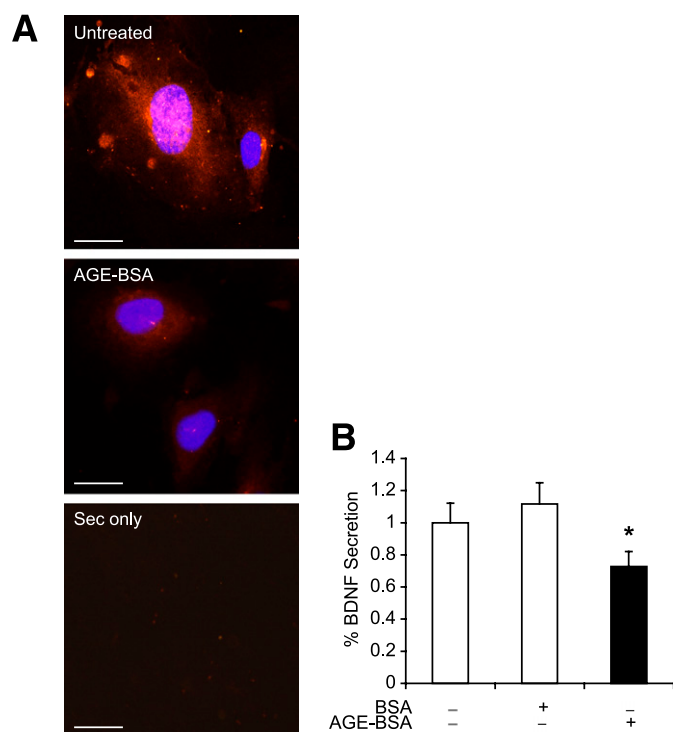


FIG. 4. BDNF expression and secretion are reduced after AGE treatment in brain endothelial cells. **A:** Representative images from untreated and AGE-treated brain endothelial cells, stained for BDNF by immunocytochemistry. BDNF is localized in perinuclear areas and at the plasma membrane in untreated cells. A reduction in BDNF staining after AGE-BSA treatment is observed compared with untreated cells. **B:** BDNF secretion is decreased after AGE-BSA treatment, as quantified by enzyme-linked immunosorbent assay on brain endothelial-conditioned media (E-CM) across treatments. * $P < 0.05$ between untreated and AGE-BSA treatment, $N = 5$ per group. (A high-quality digital representation of this figure is available in the online issue.)

and maladaptive events in diabetes (28). Degeneration of endothelial cells and smooth muscle cells in cerebral cortical arterioles was observable between 14 and 16 weeks in the STZ model of diabetes (38). Increased capillary basement membrane thickening, pericyte degeneration, and decreased cortical capillary density were observed between 4 and 8 months of diabetes in the rats with STZ-induced diabetes (39). A dramatic loss of neocortical neurons coincident with marked shortening of capillary network in the neocortical tissue was observed after 1 year of diabetes in the rat with STZ-induced diabetes by morphometric quantification (40). Taken together, a progressive depletion of microvascular neurotrophic support seems imminent in the diabetic brain. Reduced cerebrovascular BDNF support, as observed in our study, could be a molecular mechanism underlying neuronal dysfunction observed in previous studies. Our media transfer experiments with cortical embryonic neurons only provide proof of concept on how such a deficit might render neurons more vulnerable to a second insult. This cannot be unequivocally extrapolated, as yet, to BDNF dependence in the adult diabetic brain directly. Nevertheless, it is now accepted that BDNF is not only important for neural development, it (or other trophic factors) contributes to adult brain homeostasis as well. BDNF is required for survival of both developing and adult DRG neurons (41). In vivo blockade of trk receptors leads to widespread neuronal death in

adult mouse brains (42), and a conditional BDNF knock-out mouse shows disrupted striatal neuron survival and function (43). In this context, our data provide a potential mechanism that may underlie the increased neuronal vulnerability of diabetic brains.

Endothelial cells are a major source of neuroprotective BDNF protein in the brain (44–46). In addition, BDNF has unique systemic and metabotropic functions (47). Our findings also may be consistent with the clinical disease literature. Cerebral output and plasma levels of BDNF are reduced in individuals with type 2 diabetes (48–50). Because these changes occur in an insulin-dependent manner, it suggests that glycemic memory and BDNF status might be linked (49). In our study, we found that BDNF protein levels were reduced in microvessel-enriched fractions of 6-month diabetic rat cortices and that BDNF protein expression was decreased in the diabetic endothelium. Although these findings point to endothelial dysfunction as a mechanism for loss of neuroprotection in the diabetic brain, they remain reductionist, given that other cell types can secrete BDNF in the brain. The existence of compensatory mechanisms in the parenchyma that make up for the microvascular deficit cannot be ruled out. To dissect the singular contribution of the endothelium, targeted silencing of the *BDNF* gene by conditional knockout experiments or by small interfering RNA strategy will be required. Nevertheless, there is reason to believe that in the deeper cortical layers of the brain, with a high vessel-to-neuron ratio, the endothelia serve as major life-supporting repositories of trophic growth factors.

Our data suggest that BDNF is a leading candidate of cerebrovascular trophic coupling mechanisms. However, the autocrine and paracrine functions of brain endothelium are likely to include many trophic signals. Further investigation is required to dissect the interactions with other neurotrophic factors. The diabetes-induced decrease of BDNF in cerebral microvessels seems to be mediated by AGEs via activation of the ERK signaling pathway. These findings are consistent with known receptor-mediated effects of AGEs; however, nonreceptor-mediated effects of AGEs cannot be ruled out, and more investigation is required to demonstrate a direct involvement of RAGE in this pathway. Although our in vitro model of AGE-BSA treatment cannot be equated to in vivo diabetic conditions, it is useful in assessing the direct contribution of AGEs to neurotrophic reduction observed in vivo.

In conclusion, our study identified a vascular mechanism that may explain why diabetes increases risk for neurologic injury such as stroke. Strategies to preserve neurovascular trophic coupling mechanisms may lead to the development of novel preventive neuroprotective therapy in diabetes.

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No potential conflicts of interest relevant to this article were reported.

D.N. designed and conducted experiments, analyzed data, and wrote the manuscript. S.-z.G. provided technical assistance and reviewed the manuscript. K.H. performed and guided immunochemical procedures. X.W. provided diabetic brain sections for study. C.G. provided 6-month

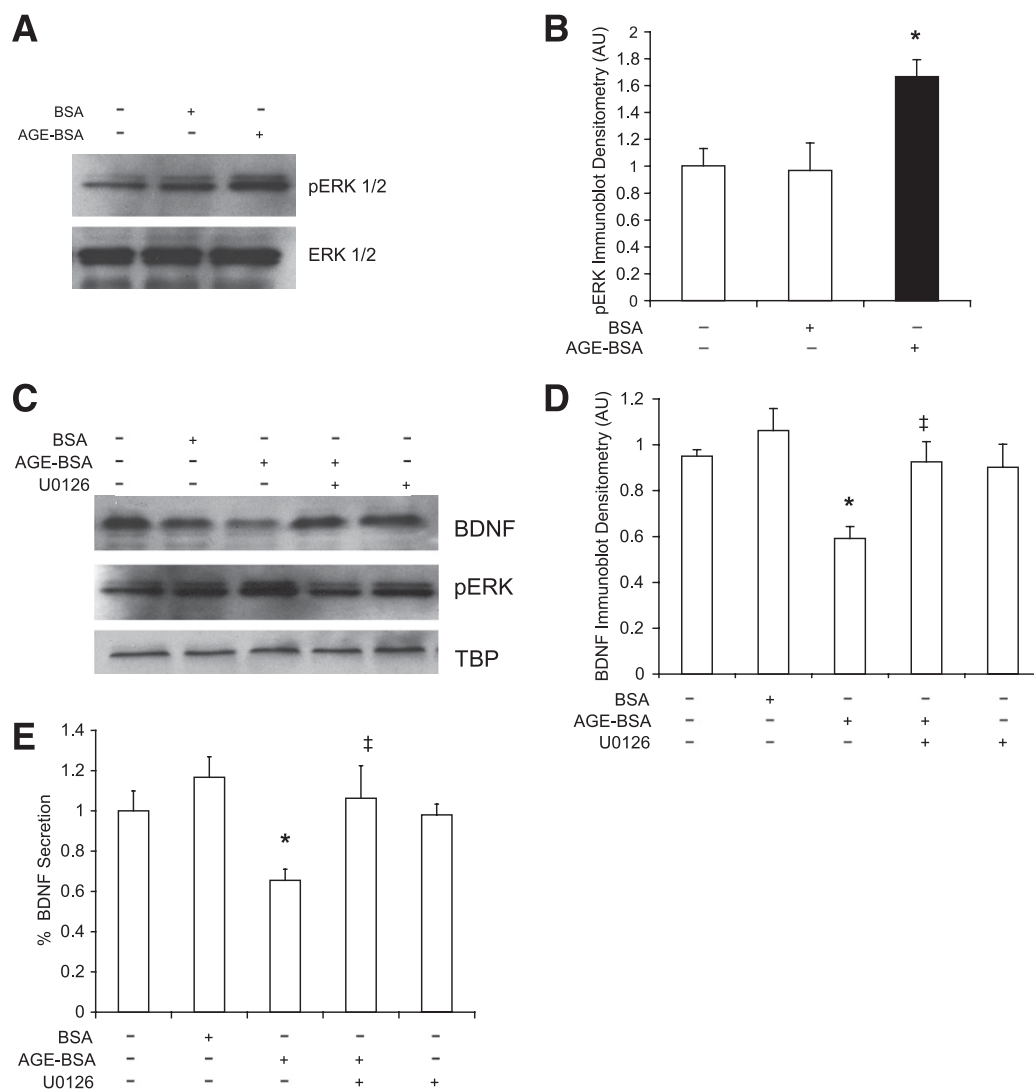


FIG. 5. Reduction of BDNF on AGE stimulation is dependent on ERK/MAP kinase signaling. **A:** Representative Western blot shows increased phospho-ERK in cells exposed to AGE-BSA for 48 h, with no change in total ERK levels. **B:** Quantitative densitometry showing that AGE-BSA-treated endothelial cells expressed significantly higher phospho-ERK levels compared with untreated cells or cells treated with normal BSA. $*P < 0.05$ between untreated and AGE-BSA treatment, $N = 3$ per group. **C:** Representative Western blot shows a coincident decrease in BDNF levels along with increase in pERK after 48 h of AGE-BSA treatment, and this effect is blocked with the MEK/ERK inhibitor U0126. **D:** Quantitative densitometry showing that the ability of AGE-BSA to suppress endothelial BDNF can be blocked by U0126. **E:** U0126 prevents the AGE-BSA-induced reduction in secreted BDNF, as quantified by enzyme-linked immunosorbent assay on brain media-CM across treatments. $*P < 0.05$ between untreated and AGE-BSA treatment. $\ddagger P < 0.05$ between AGE-BSA treatment and AGE-BSA and U0126 cotreatment, $N = 4$ per group. TBP, TATA-binding protein.

diabetic samples, contributed to discussion, and edited the manuscript. E.H.L. supervised research design, analyzed data, and co-authored the manuscript.

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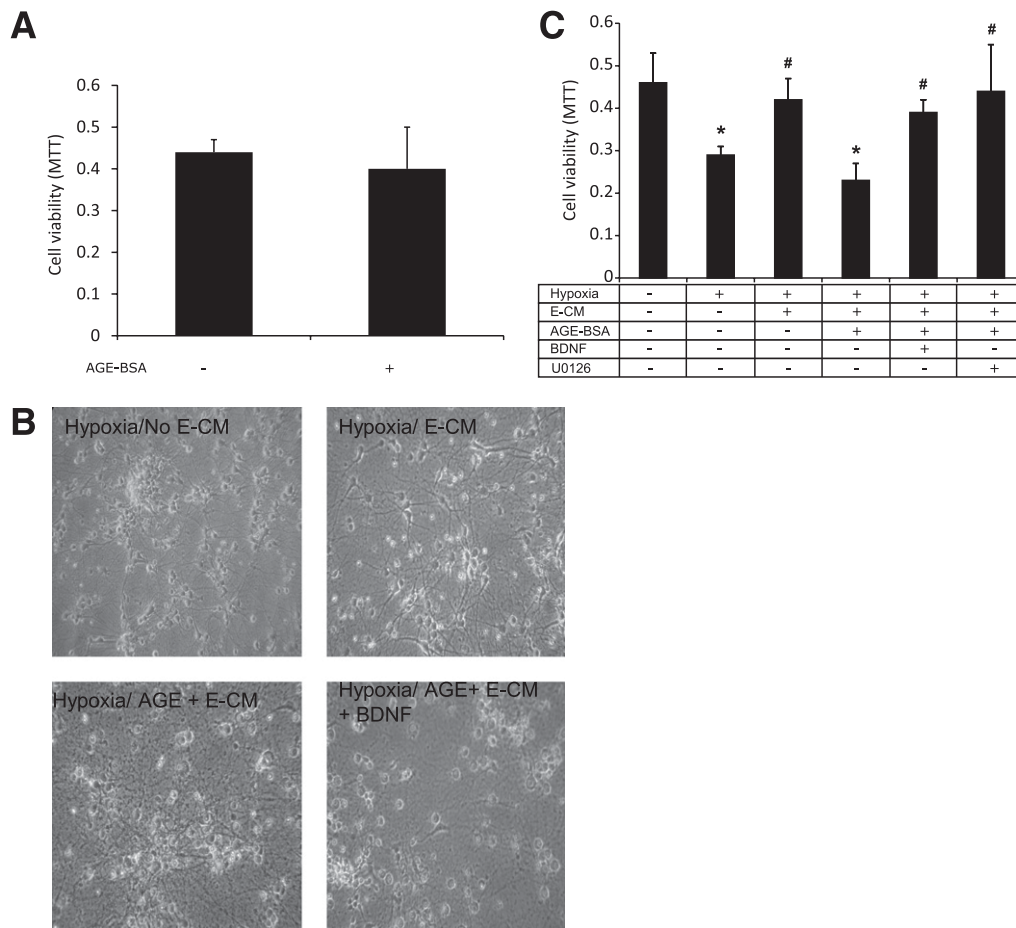


FIG. 6. BDNF-mediated neuroprotection is lost after AGE-BSA treatment of brain endothelial cells. **A:** AGE-BSA is not directly neurotoxic. Primary embryonic mouse neurons were treated with AGE-BSA (50 $\mu\text{g/mL}$) under normoxic conditions for 24 h. Cytotoxicity was assessed by MTT assay. **B:** Phase-contrast images of primary mouse neurons after 24 h of hypoxia with different treatments. Hypoxia kills neurons. Conditioned media from normal untreated brain endothelial cells reduce neurotoxicity. Conditioned media from AGE-BSA-treated brain endothelial cells are no longer neuroprotective. Addition of exogenous BDNF rescues neurons again. **C:** Quantitative comparison of MTT cytotoxicity assays. * $P < 0.05$ normoxic neurons vs. hypoxic neurons and normoxic neurons vs. hypoxic neurons in E-CM collected after AGE-BSA treatment. # $P < 0.05$ hypoxic neurons vs. hypoxic neurons in E-CM and hypoxic neurons in E-CM collected after AGE-BSA treatment vs. hypoxic neurons in E-CM collected after AGE-BSA treatment with U0126. $N = 5$ per group.

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